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Target cells in bone for parathormone and calcitonin are different: Enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces

(bone cells/cAMP)

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ABSTRACT Six populations of bone cells (populations 1–6) were obtained by sequential digestion of mouse calvaria with collagenase and trypsin. After release from the tissue, each cell population was cultured for seven days. Parathormone, but not calcitonin, elicited an increase in intracellular cyclic AMP in the cells of populations 4, 5, and 6. In contrast, both hormones elicited increases in cyclic AMP in populations 2 and 3 but had no effect on population 1. When the cells of population 2 were exposed to a Falcon tissue culture polystyrene surface for periods of time up to 5 min, many cells adhered. The nonadhering cell population contained a lesser proportion of cells responsive to calcitonin, whereas the adhering population contained a greater proportion responsive to this hormone. Conversely, when the cells of population 2 were exposed to an acid-treated nylon surface, the nonadhering cells contained a larger proportion of those responsive to calcitonin and a smaller proportion responsive to parathormone. When those cells that were enriched for calcitonin responsiveness were examined, we found an increased proportion that exhibited an asymmetric bipolar morphology. These differed from large amorphous, often binucleate, cells which predominated in those populations that responded exclusively to parathormone. These results establish that bone contains at least two types of target cells—one that responds to parathormone but not calcitonin, the other that responds predominantly to calcitonin.

Parathormone and calcitonin are peptide hormones that play major roles in bone metabolism. Both appear to utilize cyclic AMP as a second messenger (1, 2). When parathormone is added to bone maintained in culture, there occur in addition to resorption of matrix (3), decreased citrate decarboxylation (4), increased hyaluronate synthesis (5), increased lactate production (6), and decreased collagen synthesis (7). Calcitonin can temporarily block the parathormone-induced matrix resorption and the increased synthesis of hyaluronate, but it does not affect citrate decarboxylation (8).

Efforts to understand the events responsible for the metabolic changes produced by these hormones and to correlate these events with the resorption of mineral have been complicated by the heterogeneity of bone as a tissue. Several morphologically different cell types have been recognized, and these lie in a complex millieu consisting of crystalline and amorphous mineral, collagen and proteoglycans (9). Parathormone causes morphological changes in at least two types of bone cells, osteoclasts and osteocytes (10), whereas calcitonin reverses these alterations (11). It is not known at present whether the observed metabolic changes induced by parathormone and calcitonin in whole bone occur in these or other cell types.

In attempts to simplify interpretation of data, the actions of the bone-active agents have been tested directly on cells in suspension immediately after their removal from the matrix by enzymatic digestion (12), or after short-term maintenance in culture (13). Recently, by using this approach, we succeeded in separating from mouse calvaria a selected population that was enriched in cells responsive to both parathormone and calcitonin (14). Since it was likely that this population was still heterogeneous, despite the absence from it of many nonresponsive cells, it was not clear if a single cell type responded to both hormones or if instead different cell types were involved.

In the present paper we report the separation of parathormone-responsive bone cells from those bone cells that respond to calcitonin. This was accomplished through the use of mild differential digestion of mouse calvaria and by taking advantage of the selective and differential adhesiveness of the calcitonin and parathormone-responsive cells to polystyrene and nylon surfaces.

MATERIALS AND METHODS

Preparation of Cells. Cells were prepared from 2-day-old mice, strain CD-1, by a modification of the method previously described (14). 75 calvaria were digested for 20 min in 3 ml of an isotonic salt solution containing 0.1% collagenase, 0.05% trypsin, and 4 mM EDTA (14) at room temperature. Five consecutive extractions were performed. This differed from our previous method in that the number of calvaria per ml of enzyme was increased 5-fold. The cells released after each digestion were harvested and were placed in culture along with the residual calvarial remains in Falcon polystyrene flasks for 7 days. At this time the cells were tested for parathormone and calcitonin responsiveness, as shown by increased cellular cAMP levels. The monolayer cell cultures were exposed for 5 min to fresh minimal essential medium containing 10% fetal calf serum, 0.5 mM theophylline, and parathormone (0.44 U/ml) or salmon calcitonin (0.5 U/ml) singly or in combination. Cyclic AMP was measured by a modification of the method of Gilman (15) as described (14). All hormone-induced values for cyclic AMP provided in Fig. 1 and tables have been corrected for the untreated (control) level of cyclic AMP, which averaged 20 ± 5 pmol per 10^6 cells.

Binding to Polystyrene. Unless otherwise stated, all of the glassware and plasticware used in the handling and transfer of the subcultured cells were coated with serum by rinsing

in minimal essential medium containing 10% fetal calf serum. The 7-day-old cells derived from the second digestion of mouse calvaria were harvested in Ca^{++}, Mg^{++}-free Tyrode's solution containing 0.004 M EDTA, centrifuged, and resuspended in phosphate-buffered saline. One milliliter of this cell suspension was rapidly spread by swirling over the surface of a Falcon polystyrene tissue-culture petri dish (60 × 15 mm), after which the dish was allowed to sit at room temperature. At the indicated periods of time, the dishes were again swirled to suspend the nonadhering cells, which were then drawn off by pipet. The dishes were rinsed once and the rinse was added to the original supernatant. The nonadhering cells were counted in a hemacytometer, subcultured, and on the following day their responses to parathormone and calcitonin were assayed as described above. The cells bound to the polystyrene dishes were harvested by scraping with a rubber policeman in minimal essential medium with 10% fetal calf serum, centrifuged, counted, and subcultured. Hormone responsiveness was measured 24 hr later.

Binding to Nylon. Nylon vials were prepared by washing for 10 min each in carbon tetrachloride and ether, followed by treatment with 3.0 M HCl for 30 min, after which the vials were thoroughly rinsed with H_2O. Seven-day-old cells from population 2 were harvested and centrifuged as before and suspended in 2 ml of a 1:1 mixture of phosphate-buffered saline and minimal essential medium containing 10% fetal calf serum. The cell suspension was transferred to the treated nylon vial, which was tightly capped, placed on its side, and gently rolled on a reciprocating shaker at 32° for 50 min. The unbound cells were collected, placed in culture, and assayed for parathormone and calcitonin response 24 hr later.

Animals and Supplies. CD-1 mice were obtained from Charles River, Wilmington, Mass. Minimal essential medium containing Earle's salt and fetal calf serum was purchased from Gibco, Grand Island, N.Y. Polystyrene tissue culture flasks and petri dishes were obtained from Falcon, Division of Bioquest, Cockeysville, Md. Nylon vials, normally sold for radioactive assay in liquid scintillation counters, were purchased from Interex Corporation, Natick, Mass. Cyclic AMP protein kinase and protein kinase inhibitor were purchased from Sigma, St. Louis, Mo.; [8-H^3]adenosine 3′:5′-cyclic monophosphate, 30 Ci/mmol, from Amersham/Searle Corporation, Arlington Heights, Ill.; crude collagenase from Worthington Biochemicals, Freehold, N.J.; and purified lyophilized trypsin from BBL, Division of Bioquest, Cockeysville, Md. Bovine parathormone of a potency greater than 2000 U/mg was prepared in our laboratory (16). Synthetic salmon calcitonin (2500 MRC U/mg) was a generous gift of the Armour Co., Chicago, Ill.

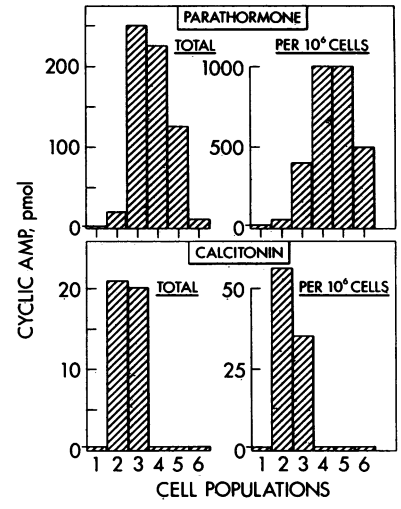


FIG. 1. Parathormone and calcitonin response in sequentially derived bone cell populations. The cells were cultured for 7 days after five sequential digestions of mouse calvaria as described in *Materials and Methods*. Population 6 was derived from cells that migrated out from the cultured calvarial remains. Changes in intracellular cAMP levels in the presence or absence of parathormone and calcitonin were measured in each population in terms of *total yield* of cyclic AMP (left) and *yield per 10^6 cells* (right).

RESULTS

Sequential Digestion of Calvaria. Typical data from one of three similar experiments are illustrated in Fig. 1. The sequential extraction of the calvaria yielded five populations of cells (populations 1–5) containing approximately 0.3, 0.6, 0.6, 1.0, and 0.7 × 10^6 cells, respectively. After 7 days in culture substantial cell division had occurred, and 1.2, 2.0, 2.4, 2.8, and 1.5 × 10^6 cells, respectively, were harvested. In addition, 0.18 × 10^5 cells had migrated out from the calvarial remains left after the fifth digestion (population 6). In each study the total yield of cyclic AMP induced by parathormone was several-fold greater than that by calcitonin. Para-

Table 1. Enrichment for parathormone or calcitonin responsiveness through selective adhesion on polystyrene

Exp.	Binding period (min)	Nonadhering cells (× 10^{-6})	Parathormone response (pmol cAMP/10^6 cells)	Calcitonin response (pmol cAMP/10^6 cells)	Relative response parathormone: calcitonin
A	0	0.50	140 ± 9	52 ± 5	2.7
	0.25	0.45	140 ± 8	14 ± 2	10.0
B	0	0.50	212 ± 8	50 ± 1	4.2
	1.5	0.17	212 ± 30	24 ± 5	8.8
	3.0	0.17	164 ± 16	22 ± 1	7.5
	5.0	0.14	114 ± 12	11 ± 3	10.3
C	0	4.0	36 ± 1	18 ± 1	2
	5	0.8	45 ± 5	5 ± 1	9

Seven-day-old bone cells derived from the 2nd digestion of mouse calvaria (population 2) were suspended in 1 ml of phosphate-buffered saline at the indicated concentration of cells per ml (zero time) and were permitted to bind to Falcon polystyrene tissue culture dishes. At the indicated times the unbound cells were collected, counted in a hemacytometer, subcultured, and assayed in quadruplicate samples for cyclic AMP production after stimulation by parathormone (0.44 U/ml) or calcitonin (0.5 U/ml). In 10 experiments, three of which are shown in this table, the relative parathormone: calcitonin response in population 2 ranged from a value of 1 to a value of 4. Data are listed ± SD.

Table 2. Preferential adherence of calcitonin-responsive bone cells to polystyrene

	No. of cells ($\times 10^{-6}$)	Parathormone response (pmol of cAMP)		Calcitonin response (pmol of cAMP)		Relative response parathormone: calcitonin
		Total	per 10^6 cells	Total	per 10^6 cells	
Initial	2.00	110 ± 5.0	55 ± 2.9	30 ± 3.7	15 ± 1.8	3.7
Nonadhering	0.67	36 ± 2.8	54 ± 4.0	0.8 ± 0.4	1.2 ± 0.5	45.0
Adhering	1.33	43 ± 1.7	32 ± 2.6	35 ± 5.0	26 ± 3.6	1.2

Seven-day-old cells derived from the 2nd digestion of mouse calvaria (population 2) were suspended in phosphate-buffered saline and permitted to bind to Falcon tissue culture polystyrene. Adhering cells and the unbound cells were assayed 24 hr later in quadruplicate samples for parathormone (0.44 U/ml) and calcitonin (0.5 U/ml) responsiveness. Data are listed ± SD.

thormone responsiveness, as measured by total cyclic AMP yield, was greatest in populations 3 and 4, but responsiveness per cell was greatest in populations 4 and 5. In contrast, calcitonin responsiveness was detected only in populations 2 and 3, with maximum responsiveness per cell in population 2. In those populations that responded to both parathormone and calcitonin, the effects of the two hormones were additive, as had been observed (14). The calvarial remains tested immediately after the fifth digestion and the relatively few cells that migrated out during the period of culture exhibited only a small total response to parathormone. Neither responded to calcitonin.

This digestion procedure, therefore, yielded populations of bone cells that responded to parathormone and not to calcitonin (populations 4–6) and populations of cells that responded to both hormones (populations 2 and 3).

Selective Adhesion of Bone Cells. The mixed response of populations 2 and 3 obtained by sequential digestion could have resulted from the presence of two different target cell types—one specific for calcitonin and one for parathormone. Conversely, one type of cell could have responded to both hormones. Consequently, we subjected population 2 to further treatment in an effort to separate the two responses.

When the cells of population 2 were transferred to Falcon dishes, although most of the cells were firmly attached within minutes, some appeared to adhere more rapidly than others. For example, after 0.25 min (Table 1, Exp. A), when 10% of the cells had adhered to the polystyrene dish, the nonadhering cells exhibited only one-fourth the specific response per cell to calcitonin. On the other hand, these same cells retained all of the specific response per cell to parathormone. Maximum separation of the parathormone and calcitonin responses occurred rapidly (Table 1, Exp. B). Higher concentrations of cells (up to 4×10^6 per ml) yielded results qualitatively similar to those obtained with a lower cell concentration (Table 1, Exp. C). Overall, this adhesion technique produced up to 12-fold separation of relative responses to parathormone and calcitonin (Tables 1 and 2, last column).

Efforts to recover the cells adhering to the polystyrene dishes were only partially successful, due to the high degree of adhesiveness of the cells, their fragility, and the small number involved. Usually only a third or less of the adhering cells could be removed by careful scraping of the dishes with a rubber policeman. These freed cells were examined for their sensitivity to calcitonin and parathormone. Table 2 lists one of four studies in which this was done. The total number of adhering cells was calculated as the difference between initial number of cells and those which did not adhere. Total hormonal response in the adhering cells was then estimated by multiplying the measured response per cell by the number of cells calculated to have adhered. The table shows that the adhering cells exhibited a substantial enrichment in response per cell to calcitonin and that they accounted for all of the responsiveness lost from the initial cell suspension.

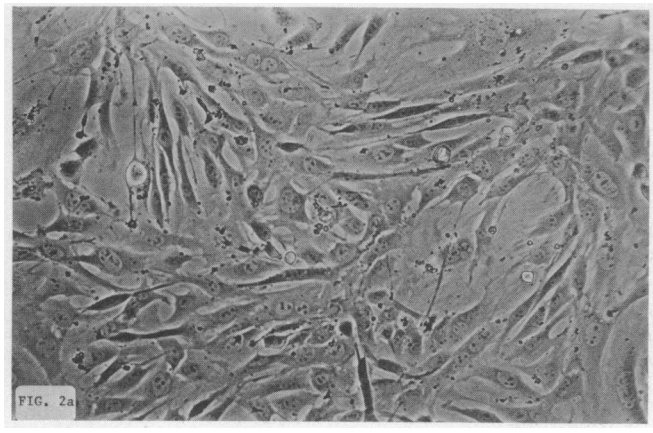
The relative adhesiveness to a nylon surface of the parathormone-sensitive and calcitonin-sensitive cells was opposite that to polystyrene. In initial tests we found that the overall rate of binding of cells to nylon was less than that to polystyrene. In subsequent studies, therefore, we used 50-min test periods. Under these conditions about 40% or less of the cells did not adhere to the surface (Table 3). In this nonadhering population the response per cell to parathormone decreased, whereas that to calcitonin increased. Consequently, the response to parathormone relative to that to calcitonin decreased from 2- to 10-fold (Table 3, last column).

Morphological Studies on Bone Cells. Immediately after

Table 3. Loss of parathormone responsiveness and enrichment for calcitonin sensitivity through selective adherence of bone cells to nylon

Exp.	Binding period (min)	Nonadhering cells ($\times 10^{-6}$)	Parathormone response (pmol cAMP/10^6 cells)	Calcitonin response (pmol cAMP/10^6 cells)	Relative response parathormone: calcitonin
A	0	0.70	100 ± 6	20 ± 3	5.0
	50	0.29	14 ± 3	28 ± 3	0.5
B	0	0.71	169 ± 10	110 ± 5	1.4
	50	0.08	105 ± 19	225 ± 17	0.45
C	0	1.60	135 ± 4	34 ± 2	3.3
	50	0.25	120 ± 12	84 ± 8	1.4

Seven-day-old cells from calvarial digestion number 2 (population 2) were exposed to a nylon surface for 50 min as described in *Materials and Methods*. The nonadhering cells were assayed in triplicate samples 24 hr later for parathormone and calcitonin responsiveness. Data are listed ± SD.



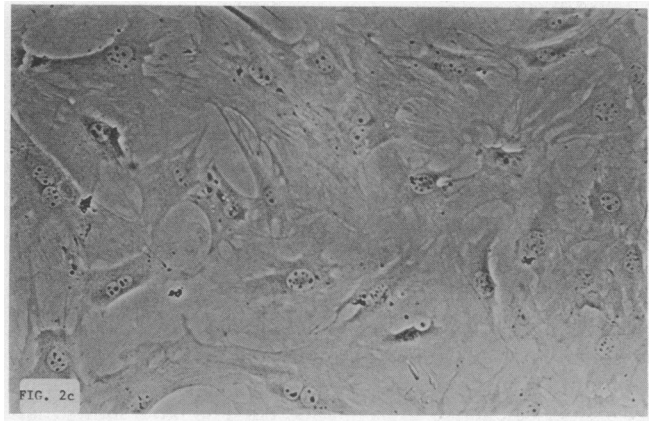


FIG. 2. Photomicrographs of different populations of bone cells obtained by sequential digestion of calvaria after 7 days in culture (phase contrast, ×500). (a) Population 1 (nonresponsive); this appears as a mixture of small compact cells in which spindle-shaped types predominate. (b) Population 2 (responsive to both parathormone and calcitonin). Three types of cells are observed: *type A*, asymmetrical cell with nucleus close to one cell membrane; *type B*, slender cells with long cytoplasmic extensions; *type C*, large, amorphous mono- and binucleate. (c) Population 4 (responsive solely to parathormone); a population consisting almost exclusively of type C cells (see b). Population 3 presented an appearance midway between populations 2 and 4; population 5 and 6 were similar to population 4. (d) Cells of population 2 adhering preferentially to polystyrene (enriched in response to calcitonin); type A cells predominate (see b). (e) Cells of population 2 nonadhering after exposure to nylon (enriched for response to calcitonin); type A cells predominate (see b).

extraction from the calvaria all of the cells were small round bodies and differed little from each other. Upon culture each population of cells exhibited somewhat different growth patterns and morphology (Fig. 2) than the preceding population. Population 1 consisted of compact spindle-shaped cells resembling those freed in the first digestion previously described (14). In populations 2 and 3, at least three morphologically distinct cell types were prominent: type A, asymmetrical cells in which the nucleus was pressed against a smooth, curving cell membrane on one side opposite a ballooning, ruffled membrane on the other; type B, slender cells with extremely long cytoplasmic extensions at either end; and type C, large amorphous and often binucleate, with many cytoplasmic processes. The latter type of cell was greater in population 3 than in 2. The cells derived from the 4th and 5th digestions of calvaria were the most morphologically homogeneous and consisted predominantly of the large amorphous cells (type C). In those fractions with enhanced response to calcitonin (e.g., polystyrene-bound, nylon-unbound) the cells contained a significantly greater proportion of the asymmetric bipolar phenotype (type B) of populations 2 and 3. In contrast, those cells surviving polystyrene adsorption were little changed in overall appearance.

DISCUSSION

Previously we reported that the sequential digestion of calvaria with collagenase and trypsin released different populations of bone cells, one of which was greatly enriched in cells that produced cyclic AMP in response to both parathormone and calcitonin (14). The increase and subsequent decrease in responsiveness to the two hormones during prolonged cul-

ture occurred together; thus it was not possible to decide whether or not a single cell type responded to both hormones. The present study has resolved this question.

By sequential digestion milder than previously reported (see ref. 14) we obtained cells that contained responsiveness for parathormone but not calcitonin. In addition, we isolated a second population of cells that could be substantially enriched in calcitonin responsiveness.

It is unknown at present whether the parathormone-responsive cells in population 2 are of the same family as those obtained in the subsequent digestions (e.g., populations 4 and 5). The morphological heterogeneity of this cell fraction makes interpretation difficult. Furthermore, it must still be determined whether the residual responsiveness to parathormone in the calcitonin-enriched population that survived nylon binding or that adhered preferentially to polystyrene (Table 2 and 3) resulted from still unseparated parathormone-sensitive cells or instead represented intrinsic response of a cell type that responds to both parathormone and calcitonin.

The possibility was considered that the digestion and/or adhesion treatments led to selective damage of one or the other type of hormone receptors rather than to nondestructive separation of cells of different hormonal sensitivity. For example, Chase (17) showed that trypsinization of kidney slices resulted in the selective loss of parathormone responsiveness. Such an event could not account for our results, however, for the following reasons. First, we measured hormone response after the cells were cultured for 7 days, a period of time during which we previously demonstrated that parathormone and calcitonin responsiveness could be regenerated in cells extracted from calvaria by collagenase and trypsin (14). Even more important, however, the studies of selective adhesion with polystyrene indicated that hormone responsiveness lost from the nonadhering cells could be recovered in the adhering population.

The molecular basis for the differential binding of cells to selected substrata is not clearly defined, although the use of adsorption to separate selected cell types is not unusual. For example, macrophages have been separated from lymphocytes by filtration through nylon fiber (18) and glass wool (19), and pancreatic beta cells have been separated from fibroblastoid cells by their differential rates of binding to Falcon polystyrene (20). This affinity for different surfaces must reflect unique properties of the cell. Specific areas of the membrane appear to be involved in attachment (21). Electron microscopic studies of cells growing on polystyrene have demonstrated that the plasma membrane does not come in contact with the substratum: attachment appears to occur through regions of close contact between plasma membrane and electron dense material which is deposited on the substrate (21).

Conceivably the preferential binding of certain cell types to various polymeric surfaces could involve specific chemical groups present on, or differences in the distribution of charged groups across the surface of, the substrate. Falcon polystyrene is reported to be a negatively charged surface designed to improve wettability and cell adhesiveness. Nylon after acid treatment likely has a mixed surface charge of dissociated carboxyl groups and amino groups. Since both plastic surfaces are equally wettable (by direct measurement), it is interesting to speculate that those cells responsive primarily to calcitonin have a preferential affinity for negative groups whereas the parathormone-responsive cells have a preferential affinity for positive charges. Such properties might relate to the location and function of these cells *in vivo* in the highly charged regions of the hydroxyapatite mineral and collagen-proteoglycan matrix.

Our results now will permit a more systematic investigation of the cellular and biochemical pathways involved in the response of bone tissue to parathormone and calcitonin as well as other bone-active agents. In such work it will be essential to identify the parathormone-responsive and calcitonin-responsive cells in terms of the well-characterized histological types of intact bone.

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